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Folate metabolic pathway single nucleotide polymorphisms: a predictive pharmacogenetic marker of methotrexate response in Indian (Asian) patients with rheumatoid arthritis

Aim: We evaluated the pharmacogenetic influence of genetic polymorphisms in folate pathway genes in Indian rheumatoid arthritis patients receiving methotrexate (MTX). **Patients & methods:** Twelve polymorphisms within nine folate pathway genes were analyzed for association with MTX response in 322 Indian rheumatoid arthritis (RA) patients and MTX pharmacokinetics in 94 RA patients. **Results:** Polymorphisms in GGH, SHMT1 and TS were associated with MTX-related adverse events while SNPs in MTHFR and RFC1/SLC19A1 were associated with MTX efficacy. TS5'UTR and SHMT1 polymorphisms were associated with higher plasma levels of MTX. **Conclusion:** Polymorphisms in folate-MTX pathway genes contribute to MTX response and affect MTX concentrations in Indian RA patients. A toxicogenetic index could identify patients who develop adverse events to MTX.

Keywords: folate metabolism • homocysteine • Indians • methotrexate • pharmacogenomics • pharmacokinetics

Methotrexate (MTX) a folic acid analog, is a first-line treatment and is the most commonly prescribed disease modifying antirheumatic drug (DMARD) for the treatment of rheumatoid arthritis (RA). It is also a key drug in most combination therapies, and a gold standard for comparing new therapies for RA. MTX is also frequently used in the management of other forms of inflammatory arthritis [1]. The efficacy and toxicity profile of MTX has been well proven in various randomized controlled trials and longitudinal cohort studies [2]; however, its use is confounded by unpredictable interpatient variability in clinical response and toxicity. Approximately 60% of patients experience good clinical response and 30% discontinue therapy due to adverse events [3–5]. Clinicians face challenges in predicting adequate disease control and adverse events in patients receiving MTX. Due to the inability to predict MTX response (efficacy and toxicity), regular blood monitoring is required in these patients. Further, additional DMARDs or costly biologic therapies are needed for MTX nonresponders. Thus MTX therapy in RA is

a dynamic process and requires subtle balance between benefits and risk, and there is a great need for better predictive markers for MTX efficacy and toxicity.

The therapeutic effectiveness and cytotoxicity of folate antagonists are both due to their inhibition of DNA and RNA synthesis. Folate metabolism is the major target of MTX (Figure 1). However, the exact mechanism by which MTX modulates inflammation in RA is still unclear. It is thought that the anti-inflammatory effects mediated by adenosine release may be more important than the antiproliferative effects [6,7]. MTX enters the variety of cells including RBCs, WBCs, hepatocytes, synoviocytes through reduced folate carrier 1 (RFC1) or SLC19A1. Once inside the cell, MTX is intracellularly converted to active MTX polyglutamates (MTXPGs) by folypolyglutamate synthase (FPGS) by sequential addition of glutamate residues. MTXPGs directly inhibit target enzymes of the folate pathway, like, dihydrofolate reductase (DHFR), thymidylate synthase (TS) and further inhibit key enzymes involved in *de novo* purine syn-

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Figure 1. Intracellular methotrexate metabolic pathway. Figure illustrates schematic representation of the intracellular folate biosynthetic pathway and related pathways. Enzymes involved in different pathways are denoted in bold. Transporter/enzyme polymorphisms genotyped in the current study are highlighted in bold with red font. Transporters: ABCB1 and ABCC1–4: Adenosine triphosphate-binding cassette (ABC) transporters; hFR: Human folate carrier; RFC-1: Reduced folate carrier1. Enzymes: ADA: Adenosine deaminase; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase/IMP cyclohydrolase; CBS: Cystathionine- β -synthase; CL: Cystathionine lyase; DHFR: Dihydrofolate reductase; FPGS: Folylpolyglutamyl synthase; GART: Glycinamide ribonucleotide formyltransferase; GGH γ : Glutamyl hydrolase; MS: Methionine synthase; MTHFR: Methylene tetrahydrofolate reductase; MTHFD1: Methylene tetrahydrofolate dehydrogenase; MTRR: Methionine synthase reductase; SHMT: Serine hydroxymethyltransferase; TS: Thymidylate synthase. Metabolites: ADP: Adenosine diphosphate; AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; CH3: Methyl group; DHF: Dihydrofolate; dTMP: Deoxythymidine-5'-monophosphate; dUMP: Deoxyuridine-5'-monophosphate; FAICAR: 10-formyl-AICAR; Imp: Inosine monophosphate; GAR: Glycinamide ribonucleotide; MTX: Methotrexate; MTX-PG: methotrexate polyglutamates; THF: tetrahydrofolate. Vitamins: B $_2$, B $_6$ and B $_12$ are cofactors in the pathway.

thesis such as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC) and GART. This causes accumulation of adenosine, which has anti-inflammatory activity [7]. Other folate enzymes, such as methylenetetrahydrofolate reductase (MTHFR), serine hydroxymethyltransferase 1 (SHMT) and enzymes in the one carbon pool [methionine synthase (MS) and methionine synthase reductase (MTRR)] are not directly inhibited by MTX, but their expression level may contribute to the antifolate effects of MTX through subtle alterations in the folate pools [8]. γ -glutamyl hydrolase (GGH), catalyzed the conversion of MTXPGs back to MTX and this MTX is then rapidly efflux from the target cell by ATP-binding cassette (ABC) transporters, predominantly ABCC1-ABCC4 and ABCG2. Efficiency or activity of these MTX transporters and metabolizing

enzymes could be influenced by genetic variations in the genes that encode them, which in turn would affect the net therapeutic effect of the drug.

Significant interindividual and interethnic differences are observed in the dose of MTX required to achieve antirheumatic effects [9]. Recent studies have increasingly recognized the association of single-nucleotide polymorphisms (SNPs) of the folate metabolic pathway as genetic predictors of MTX response [1,10,11]. However many of these findings still require replication, and the inclusion of representative world populations in these studies is still incomplete [1,12]. MTX pharmacogenetics studies have largely been performed on Western and East Asian population and very few reports with small sample size are available on Indian (Asian) RA population [13–18]. In the present study, we applied

a pathway-driven approach in order to evaluate the contribution of SNPs in folate metabolic pathway genes as a pharmacogenetic marker of MTX response (both toxicity and efficacy) in Indian (Asian) patients with rheumatoid arthritis. In addition, we performed an in-depth pharmacokinetic analysis including plasma MTX, 7OH-MTX and homocysteine (Hcy) measurements, coupled with detailed clinical follow-up data.

Patients & methods

Study design & clinical management

This was cross-sectional study conducted at Center for Rheumatic Diseases (CRD), Pune. CRD is a popular regional center with a high patient volume (70–90 patients daily) [19]. CRD maintains a comprehensive patient database since 1998, including data from a standardized rheumatology case record form (clinical and adverse events) and laboratory investigations for all patient visits. All patients are carefully questioned from an *a priori* list of possible drug adverse events and encouraged to provide any other information at every clinical examination visit.

Patients were screened to determine the eligibility criteria for inclusion into the study. To be eligible, the patients (age ≥ 18 years) had to meet the American College of Rheumatology (ACR) revised classification criteria for RA [20] and had to have received MTX for at least 3 months. All patients included in this analysis began treatment with a regimen of oral MTX at a dosage of 7.5 mg weekly, with the dosage increasing to a maximum of 20 mg weekly. In the event of insufficient clinical response at each follow-up visit, the MTX dosage was increased in a stepwise fashion, increasing 2.5 mg every 4 weeks to 20 mg weekly. Thus, the dose of MTX and any adjustment in the dose was standardized. Folic acid supplements were not prescribed as a routine care. However, the majority of patients (>80%) was believed to have consumed folic acid intermittently either as a supplement (1–3 mg daily) combined with oral iron to treat moderately severe anemia (Hb <9 gm/dl) or a prophylactic (5 mg weekly with an interval of 2–3 days post MTX administration) with higher doses of MTX (>12.5 mg weekly). In case of adverse reactions, MTX was continued at the lowest tolerated dosage. In some patients showing adverse reactions MTX was stopped and restarted with lowest tolerable dose. In case of intolerance, MTX was given subcutaneously. If MTX was not tolerated at all, then the patient was offered an alternate treatment. Other concomitant medications included NSAIDs, corticosteroids and additional DMARDs.

Patient selection

A total of 403 RA patients on MTX were screened (according to clinic attendance) from May 2007 to

January 2008 (36 weeks). Out of these 322 RA (278 females) patients having regular follow-up and completing at least 1 year of MTX were enrolled into the present study. All subjects were enrolled sequentially from the clinic. Blood samples were collected and MTX-related toxicity and genotype analysis was done in these patients. From these 322 patients; 217 patients were analyzed for MTX-related efficacy analysis and 94 patients were selected for pharmacokinetic analysis. A systematic representation of the study design is shown as a flow chart in Figure 2. The study protocol was approved by the Institutional Ethics Committee and informed consent was obtained from all the patients participating in the study.

Clinical assessments

Demographic information was collected from each patient at the time of enrollment in the study. All patients underwent a monthly evaluation. The clinical efficacy of MTX during treatment period was assessed by change from the baseline in ACR (American College of Rheumatology) core set measures: Tender Joint Count, Swollen Joint Count, Pain in joints Visual Analog Scale (VAS), Health assessment questionnaire (HAQ) score, Patient Global Assessment of Disease Activity, Physician Global Assessment of Disease Activity, Morning stiffness, ESR/CRP (C-Reactive Protein) (Normal range ESR [Westergren]: Male <20 mm in first hour, Female <30mm in first hour; CRP [Nephelometry] <6 mg/l). Responders were classified as patients having ACR 50 response at 12 months and having $\geq$ ACR 20 response at 6 months. ACR 20 and 50 responses were calculated as per ACR guidelines [20].

Toxicity was assessed by monitoring for adverse events (AE), including physical examination and laboratory parameters for safety evaluation. At all follow-up visits, the investigator asked about any new complaints that were not existing since last follow-up, and any such complaints were recorded and entered into the clinical record form (CRF). Pre-existing symptoms that increased in severity or frequency during the treatment period were also noted and entered on the CRF. Any one or combinations of the following events were noted as 'overall AE'. MTX-related AE were defined as Gastrointestinal (GIT) which include, upper GIT (nausea, vomiting, acidity, dyspepsia, anorexia, gastritis), lower GIT (diarrhea, constipation anal burning) and pain in the abdomen (stomatitis, pancreatitis); Hepatic (Serum SGPT, SGOT >twice the upper limit of normal); Bone marrow (leucopenia <3500/mm³, thrombocytopenia <100,000 mm³, anemia, hemoglobin 20% drop from baseline values); skin rash/aggravated nodules; Mucositis (oral ulcers, glossitis, chelitis,

Figure 2. Patient enrollment and study design.

MTX: Methotrexate.

dry mouth); Hair loss, CNS symptoms (headache, drowsiness, fatigue and discomfort) and any other known MTX-related AE which has been documented in drug literature. Some patients had more than one type of adverse event. Leukocyte counts, hemoglobin and liver enzyme concentrations were measured on the day of each study visit, using standard laboratory methods. The clinical action taken to address MTX toxicity was also recorded; this consisted of ‘dose maintained with patient under observation’, ‘dose reduced’, ‘route of administration changed’, ‘dose temporarily interrupted’ or ‘dose permanently discontinued’.

Laboratory measurements

Postconsent, a peripheral blood sample (4–5 ml) was drawn from each subject, and genomic DNA was extracted using Miller’s protocol [21]. A total of 12 polymorphisms in nine genes of MTX metabolism (including transporters) were studied (Figure 1). Poly-

morphisms within the candidate genes were selected from the previous reports in the literature based on their clinical and functional importance. The polymorphisms studied were: *MTHFR* (rs1801131, rs1801133), *TS* (5’UTR repeat and 3’UTR deletion [rs34489327]), *RFC1* (rs1051266), *MS* (rs1805087), *SHMT1* (rs1979277), *MDR1* (rs1045642, rs1128503), *GGH* (rs3758149), *ATIC* (rs2372536) and *MTRR* (rs1801394). Genotyping was performed using PCR-RFLP technique and standard real-time TaqMan allelic discrimination assay as described previously [22–25]. Samples containing variants were reanalyzed to ensure the accuracy of the method. There was 100% reproducibility. The observed genotype frequencies of the studied SNPs did not deviate significantly from Hardy–Weinberg equilibrium proportions.

Plasma MTX and its main metabolite 7-OH MTX levels were determined using HPLC with postcolumn

photo-oxidation- fluorescence detection [26,27]. Subjects were given their weekly dose of MTX after an overnight fast. A 2–3-ml blood sample was collected at 0 h before MTX administration for baseline values, at 2 h and at 8 h after MTX administration. Plasma samples and RBCs were separated within 15 min of collection and stored at -70°C until analyzed. Baseline plasma Hcy was estimated in all these patients by HPLC [28,29].

Statistical analysis

All normally distributed data are expressed as the mean $\pm$ SD while non-normally distributed data are expressed as the median $\pm$ interquartile range. All categorical data are expressed as frequencies and percentages. Differences in baseline characteristics were analyzed by Student's *t*-test for normally distributed continuous variables or chi-square test/Fischer's exact test for dichotomous variables. For efficacy and toxicity response, the genotype differences between responders and nonresponders or between patients with and without AEs were tested by 3 $\times$ 2 cross-tabulations for

each genotype, and by 2 $\times$ 2 cross-tabulations for each possible combination of homozygote and heterozygote genotypes, with a chi-square test. While Fischer's exact was applied for lower number of observations in any of the genotype group ($n < 20$).

The association of the presence of risk genotype with the occurrence of adverse events or efficacy was represented as odds ratios (ORs). If needed, the minor allele homozygous genotypes with low patient number were combined with heterozygous genotypes for analysis purpose. The homozygous risk genotypes which showed statistically significant association ($p < 0.05$) with adverse events were taken into consideration as risk genotypes for calculating toxicogenetic index as described earlier [25]. The presence of the four homozygous risk genotypes was summed as a composite index to constitute a TI for each patient (index range 0–4). Statistical associations were assessed with a multiple logistic regression model, with the occurrence of adverse events as the outcome variable and the TI as the predictor variable. Additionally, possible confounding variables considered to be clinically important were included in the multiple

Table 1. Clinical characteristics and profile of methotrexate-related adverse events of the 322 rheumatoid arthritis patients enrolled in the study.[†]

Clinical features	Values
Age (years)	43.8 $\pm$ 10.4
Sex, n (%) women	278 (86)
Duration of disease (years)	5.6 $\pm$ 4.9
Dosage of MTX (mg/week)	15.0 $\pm$ 3.9
Rheumatoid factor positive (%)	253 (79)
Number of years receiving MTX	2.4 $\pm$ 1.7
Folic acid supplementation (<10 mg/week), n (%)	269 (84)
Concurrent DMARD, n (%)	83 (26)
Concurrent NSAID, n (%)	67 (21)
Concurrent steroids, n (%)	66 (20)
Overall AE, n (%)	170 (53)
GIT AE, n (%)	101 (31)
Upper GIT	96 (30)
Lower GIT	6 (1.9)
Pain in abdomen	19 (6)
Hepatic AE, n (%)	69 (21)
Mucositis, n (%)	43 (13.4)
Bone marrow, n (%)	24 (7.5)
Hair loss, n (%)	14 (4.3)
Central nervous system side-effects, n (%)	7 (2.2)

[†]Except where indicated otherwise, values are mean $\pm$ standard deviation. Some patients had more than one type of side-effect. AE: Adverse event; DMARD: Disease-modifying antirheumatic drug; GIT: Gastrointestinal tract; MTX: Methotrexate; NSAID: Nonsteroidal anti-inflammatory drug.

logistic regression models for both efficacy and toxicity, including age, sex, duration of disease, MTX dose (mg/week), number of years of treatment with MTX, corticosteroid use, DMARD use and NSAID use. Genotype-MTX response analyses were performed using SPSS software (version 10.0 for Windows; SPSS, IL, USA) and Graph Prism (version 4.0 for Windows; CA, USA). $p < 0.05$ was considered significant. Based on the nine genes studied, the threshold p value for significance after Bonferroni correction for multiple testing was 0.006. p values between 0.05 and 0.006 are reported, but should be considered marginal.

Since PK end points were non-normally distributed according to D'Agostino-Pearson omnibus test and Shapiro-Wilk normality test (determined using Graph pad software, CA, USA), group differences were analyzed nonparametrically by use of the Wilcoxon rank sum test to compare binary groups (e.g., GG + GT vs TT) and Kruskal-Wallis test to compare three groups of genotype for each polymorphism (e.g., GG vs GT vs TT). A p -value of less than 0.05 was used to indicate significance. Genotype-PK correlations were determined using R-statistical analysis software [30].

Results

MTX-related adverse events findings

The patient's demographic and clinical data along with the MTX-related adverse events data are summarized in Table 1. A total of 170 patients (53%) presented with the adverse events.

Signs of hepatotoxicity (serum SGPT, SGOT >twice the upper limit of normal) were observed in 69 patients (21%). Seven patients had an elevation of SGPT that was more than twice the upper limit of normal values and one patient exhibited hematopoietic toxicity. One patient suffered from severe skin rash presumably due to MTX allergy.

In 100 (59%) of the 170 patients demonstrating adverse events, the MTX dose was maintained and the patients were observed. MTX use was temporarily stopped and restarted in two patients (one patient with thrombocytopenia and one with gastrointestinal AE). MTX was reduced and continued in 58 patients (34%). MTX dose was skipped in three patients (one patient with thrombocytopenia and two with an elevation of SGPT that was more than twice the upper limit of normal value). MTX dose was discontinued in six patients (two patients hypersensitive to MTX, one patient with severe pancreatitis, two patients with severe gastrointestinal intolerance and one with very high SGPT that was more than twice the upper limit of normal value).

The adverse event frequency was 69% in patients receiving MTX only (74 of 106 patients), 61% in patients receiving MTX with a concurrent DMARD (50 of 82 patients), 36% in those receiving MTX with corticosteroids (20 of 56 patients), 33% in those receiving MTX with an NSAID (21 of 64 patients) and 36% in those receiving MTX with an NSAID, corticosteroid and a DMARD (5 of 14 patients). There was no indication of an association in the frequency of AE among these groups ($p > 0.05$).

The genotype frequencies of these patients for MTX adverse events are given in Supplementary Table 1. The SHMT1 C allele was associated with the occurrence of gastrointestinal (pain in abdomen) AE (CC or CT = OR: 6.9; $p < 0.0001$). Hepatic AE was associated with the GGH 401TT genotype (OR: 2.1; $p = 0.007$). The occurrence of bone marrow adverse events was associated with the TS 5'UTR2R/2R genotype (OR: 4.9; $p = 0.001$). One risk genotype was not strongly associated with individual adverse events but was associated with an increased occurrence of overall AE (TS 3'UTR 6bp/6bp genotype, OR: 2.8; $p = 0.0005$).

Table 2. Occurrence of adverse events by risk genotype.†

Genotype	Hepatic	Bone marrow	Pain in abdomen	Overall adverse events
TS 5'UTR, 2R/2R vs 3R/3R or 2R/3R‡	1.5 (0.8–2.9)	4.5 (1.9–10.7; $p = 0.001$)	0.9 (0.2–3.2)	1.8 (1.0–3.4; $p = 0.04$)
TS 3'UTR, 6bp/6bp vs 6bp/0bp or 0bp/0bp	1.9 (1.0–3.5; $p = 0.04$)	2.1 (0.9–5.2)	1.1 (0.3–3.3)	2.8 (1.6–5.2; $p = 0.0005$)
GGH C401T, TT vs CC or CT	2.1 (1.2–3.6; $p = 0.007$)	1.2 (0.5–2.8)	1.2 (0.5–3.0)	1.7 (1.1–2.6; $p = 0.029$)
SHMT1 C1420T, CC or CT vs TT§	1.5 (0.8–2.7)	0.5 (0.2–1.6)	6.9 (2.5–18.8; $p < 0.0001$)	1.5 (0.9–2.5)

Values that passed the the threshold p -value or close to for Bonferroni correction for multiple testing are highlighted in bold font.

†Values are odds ratio (95% CI). A total of 170 patients experienced side-effects.

‡Six patients carrying TS 5'UTR alleles with >three repeats were excluded from analysis.

§Ten patients showing weak signal for SHMT1 C1420T Taqman assay were excluded from analysis. The raw data of the genotype distribution in each adverse event are presented in Supplementary Table 2.

Results for these polymorphisms which demonstrated significant associations are presented in [Table 2](#) and the raw data of the genotype distribution in each adverse event is presented in [Supplementary Table 2](#). The SNPs in *MTHFR*, *RFC1*, *MS*, *MDR1*, *MTRR* and *ATIC* were not associated with MTX-related AE in our RA population.

Other factors associated with increased occurrence of hepatic and bone marrow AE were duration of MTX administration ($p = 0.001$). None of the other factors like age, sex, disease duration, DMARD, NSAID and corticosteroid administration, CRP and RF were associated with any of the adverse events.

Association of MTX-related adverse events, dose & risk genotypes

It was observed that 29% (49 of 170 patients) of the patients having overall AE were receiving ≥ 15 mg/week MTX dose as against only 3% of patients in non-AE were receiving ≥ 15 mg/week MTX dose ($p = 0.00001$). However, MTX dose was not associated with any organ-specific adverse event. There was no association observed for any of the homozygous risk genotype and MTX toxicity dose, except for GGH 401 TT. It was observed that the patients having GGH 401 TT genotype were receiving higher doses of MTX (≥ 15 mg/week; $p = 0.01$). In six patients where MTX discontinuation was required; one patient had carried risk genotype TS5'UTR 2R/2R, one had GGH 401 TT, one with TS 3'UTR 6bp/6bp, one patient with SHMT1 CC, one patient had carried combination of two risk genotype TS 5'UTR 2R/2R- TS 3'UTR 6bp/6bp while one patient did not carry any of the risk genotype mentioned above. In some patients, MTX was stopped and restarted. One of such patient had TS 5'UTR 2R/2R- TS 3'UTR 6bp/6bp- GGH 401 TT and the other patient carried GGH 401 TT. The toxicogenetic index ranged from 0 to 4. An increased toxicogenetic index value correlated with an increased incidence of adverse events ($p = 0.00001$). Contribution of the toxicogenetic index to the occurrence of AE in Indian (Asian) patients with rheumatoid arthritis receiving MTX is represented in [\(Figure 3\)](#). We also calculated the incremental index value to find out the fold increase in the occurrence of AE within the 0–4 toxicogenetic index in our RA patients. It was observed that each incremental index value (1 unit, for a total of 4 units in our RA population) resulted in a 1.33-fold increased likelihood of presenting with an adverse event [\(Figure 3B\)](#). As the toxicogenetic index increases the percentage of the patients AE increases. Patients with an index of 4 were 2.63-times more likely to present with an adverse event compared with those with an index of 0 ($p = 0.0001$). This observation remain consistent even

when patients were divided in low (<15 mg/week) and high (≥ 15 mg/week) MTX dosage groups. In the group of patients who received MTX only ($n = 106$; no concurrent corticosteroids, DMARDs or NSAIDs), this association of adverse events with the toxicogenetic index remained statistically significant ($p = 0.0001$).

Association of MTX efficacy, dose & genetic polymorphism

The clinical characteristics of patients in efficacy analysis are represented in the [Supplementary Table 3](#). It was observed that at 12 months patients carrying at least one *MTHFR* 1298 A allele (AA-AC) were more likely to have better MTX efficacy relative to those with *MTHFR* 1298 CC (OR: 2.6; 95% CI: 1.1–5.8; $p = 0.02$). Similarly, those with an *RFC1* 80 A allele (AA-GA) had better response to MTX than those with the *RFC1* 80 GG genotype (OR: 2.2; 95% CI: 1.1–4.4; $p = 0.03$) [\(Table 3\)](#). None of the other studied SNPs were associated with MTX efficacy in our RA population.

The demographic and clinical factors like age, sex, disease duration, CRP, RF, duration of MTX administration, NSAID, DMARD and corticosteroid administration did not show any association with MTX efficacy (data not shown). No dose specific effect was observed on MTX efficacy when responders were divided based on low (<15 mg/week) and high (≥ 15 mg/week) MTX dose (data not shown). None of the SNPs in responders and non-responders were associated with MTX dose. It was observed that MTX efficacy was neither associated with any of the organ specific nor with the overall AE.

Genotype-MTX PK association analysis

There was no gender effect on plasma levels of MTX, 7OH MTX and Hcy. To understand if there was a dose-dependent increase in MTX, 7-OH MTX or Hcy plasma levels; 94 patients were divided in two MTX dosage groups: low (<15 mg/week; $n = 32$) and high (≥ 15 mg/week; $n = 62$), and plasma levels for MTX, 7-OH MTX and Hcy were compared between these two dosage groups. The plasma level of MTX at 2 h was significantly higher in patients receiving ≥ 15 mg/week dose (<15 mg/week vs ≥ 15 mg/week; 250.97 ± 198.32 vs 394.39 ± 283.13 pmoles/l; $p = 0.003$, [Figure 4A](#)). Incorporating genotype information, patients with the TS5'UTR 2R/2R genotype (18 of 94 patients) had significantly high plasma levels of MTX at 2 h (2R/2R vs 2R/3R + 3R/3R: 518.2 ± 417.23 vs 298.0 ± 193.86 pmoles/l; $p = 0.001$) than patients with 2R/3R and 3R/3R genotypes [\(Figure 4B\)](#). This observation remained significant when patients were subgrouped in low and high MTX dosage groups [\(Figure 4C\)](#). Although, marginally significant, patients with pres-

ence of *SHMT1* 1420TT genotype had lower MTX plasma levels at 2 h in both MTX dose group ($p = 0.05$). This observation was more significant in patients receiving low MTX dose as compared with high MTX dose ($p = 0.02$; Figure 4D). No association was observed between any studied SNPs and plasma levels of MTX 8 h and 7OHMTX at different time points.

We also evaluated baseline plasma Hcy in these patients. There was no effect of MTX dose on plasma Hcy levels. We observed a significant association between the *RFC1* nonsynonymous SNP rs1051266 (G>A) and plasma Hcy levels. Patients with at least one G allele demonstrated significantly higher plasma Hcy (AA vs GA + GG; 7.98 ± 3.25 vs 12.25 ± 7.80 $\mu\text{M/l}$; $p = 0.035$) as compared with patients with the AA

genotype (Figure 4E). No significant association was observed between any other studied SNPs with plasma Hcy concentration.

Correlation of PK with MTX response (toxicity & efficacy)

The plasma levels of MTX, 7 OH MTX at 2 and 8 h and baseline Hcy was calculated in patients showing MTX-related AE. There was no significant association of plasma levels of MTX, 7OH MTX and Hcy at given time point either with any organ-specific AE or overall AE. However we observed that as the MTX dose increases (from lowest dose group 3–5 mg to highest dose group 20 mg), there is an increase in the ADR and toxicogenetic index. This trend was prominent in plasma MTX levels measured at 2 h. Figure 5 demonstrates correlation of plasma levels of MTX measured at 2 h with AE and toxicogenetic index. Correlation of plasma Hcy levels with AE and toxicogenetic index (greater than or equal to greater than or equal to Figure 6) revealed that all the patients having high plasma Hcy levels (>15 $\mu\text{moles/l}$) exhibited presence of one or more AE. Forty five percent of the patients with high Hcy levels carried high toxicogenetic index (T.I. 3) with AE (OR: 6.8; 95% CI: 1.9–24.3; $p = 0.003$) as against those having <15 $\mu\text{moles/l}$ of plasma Hcy.

There was no difference in the mean plasma levels of MTX, 7OH MTX and Hcy at given time points between responders and nonresponders indicating lack of correlation with MTX efficacy.

Discussion

Although MTX is a first line of treatment for RA worldwide, wide interpatient variation in treatment response and AE complicates its use. This interpatient variation could be attributed to variability in the expression or activity of the genes within the folate-MTX metabolic pathway that could alter the pharmacokinetics and influence MTX response. There are sparse data on pharmacogenetics of MTX in Indian (Asian) patients with RA. To address this void in the literature, in the present study we comprehensively evaluated SNPs in nine potentially significant folate-MTX metabolic pathway genes in Indian (Asian) RA patients receiving MTX for association with MTX response (efficacy and toxicity) and pharmacokinetics end points. Our study demonstrated that *GGH* promoter SNP rs3758149; *SHMT1* nonsynonymous SNP rs1979277; *TS* 5'UTR variable tandem repeats and *TS* 3'UTR deletion was associated with MTX-related AE either organ specific or overall AE (Table 2). While nonsynonymous SNPs in *MTHFR* rs1801131 and *RFC1/SLC19A1* rs1051266 were associated with MTX efficacy (Table 3). We were unable to establish any significant associations or rep-

Figure 3. Contribution of the toxicogenetic index to the occurrence of adverse events in Indian (Asian) patients with rheumatoid arthritis receiving methotrexate.

(A) The toxicogenetic index was calculated as the sum of four risk genotypes carried by the patient (*TS* 5'UTR *2R/2R; *TS* 6bp/6bp; *GGH*-401 TT; *SHMT1* CC). The number (%) of patients is given for each incremental unit of the index. (B) Percentage of patients with an adverse event in response to treatment with methotrexate in relation to increasing toxicogenetic index. A total of 170 patients presented with an adverse event.

Table 3. Frequency distribution of 12 single nucleotide polymorphisms in folate metabolism associated with ACR 50 response.

Polymorphism	n = 217 genotype frequency	ACR 50 response	
		Responders (n = 49)	Nonresponders (n = 168)
MTHFR C677T			
CC + CT	166 + 49 (0.99)	38 + 10 (0.98)	128 + 39 (0.99)
TT	2 (0.01)	1 (0.02)	1 (0.01)
MTHFR A1298C			
AA + AC	58 + 95 (0.70)	12 + 29 (0.84)[†]	46 + 66 (0.67)
CC	64 (0.30)	8 (0.16)	56 (0.33)
TS5UTR			
3R/3R + 2R/3R	80 + 89 (0.79)	14 + 21 (0.73)	75 + 59 (0.81)
2R/2R	44 (0.21)	13 (0.27)	31 (0.19)
TS3UTR			
0bp/0bp + 6bp/0bp	49 + 111 (0.74)	9 + 28 (0.76)	40 + 83 (0.73)
6bp/6bp	57 (0.26)	12 (0.24)	45 (0.27)
MDR1 C3435T			
CC + CT	36 + 106 (0.65)	9 + 21 (0.61)	27 + 85 (0.67)
TT	75 (0.35)	19 (0.39)	56 (0.33)
MDR1 C1236T			
CC + CT	40 + 108 (0.68)	9 + 22 (0.63)	31 + 85 (0.69)
TT	69 (0.32)	18 (0.37)	51 (0.31)
RFC1 G80A			
AA + GA	35 + 95 (0.60)	11 + 25 (0.73)[‡]	24 + 70 (0.56)
GG	87 (0.40)	13 (0.27)	74 (0.44)
MS A2756G			
AA + AG	97 + 88 (0.85)	20 + 22 (0.86)	77 + 66 (0.85)
GG	32 (0.15)	7 (0.14)	25 (0.15)
MTRR A66G			
AA + AG	54 + 104 (0.73)	13 + 22 (0.71)	41 + 82 (0.73)
GG	59 (0.27)	14 (0.29)	45 (0.27)
GGH- 401			
CC + CT	31 + 87 (0.54)	7 + 19 (0.53)	24 + 68 (0.55)
TT	99 (0.46)	23 (0.47)	76 (0.45)
ATIC C347C[§]			
CC + CG	49 + 108 (0.74)	11 + 25 (0.73)	38 + 83 (0.74)
GG	55 (0.26)	13 (0.27)	42 (0.26)
SHMT1 C1420T			
CC + CT	7 + 51 (0.28)	2 + 12 (0.29)	5 + 39 (0.26)
TT	159 (0.73)	35 (0.71)	124 (0.74)

[†]OR: 2.6; 95% CI: 1.1–5.8; p = 0.02.

[‡]OR: 2.2; 95% CI: 1.1–4.4; p = 0.03.

[§]Four patients carrying TS 5UTR alleles with >three repeats.

[§]Five patients showing weak signal for ATIC C347C Taqman assay were excluded from analysis.

Statistically significant differences in frequency of genotypes between responders and nonresponders are highlighted in bold.

licate previous associations of SNPs within the genes *MS*, *MDR1*, *MTRR* and *ATIC* with MTX efficacy or toxicity in this study [1,9,11,31]. PK analysis revealed that presence of *TS5'UTR* 2R/2R genotype and at least one *SHMT1* 1420C allele was associated with higher

plasma levels of MTX at 2 h in our RA patients. A significant correlation was found between the *RFC1* nonsynonymous SNP rs1051266 (G>A) and plasma Hcy levels in these RA patients.

In vitro and *in vivo* studies have shown SNPs in the

Figure 4. Association of single nucleotide polymorphisms in folate metabolism with methotrexate and homocysteine pharmacokinetics (for [A–D], please see above; for [E], see facing page). (A) Box plot for plasma MTX levels measured at 2 h in patients receiving low and high MTX dose. (B) Box plot for association of *TS5'UTR* SNP with plasma MTX levels measured at 2 h. (C) Box plot for association of *TS5'UTR* SNP with plasma MTX levels measured at 2 h in patients receiving low and high MTX dose. (D) Box plot for association of *SHMT1* SNP (rs1979277) with plasma MTX levels measured at 2 h in patients receiving low and high MTX dose. (E) Box plot for association of *RFC1* SNP (rs1051266) with plasma homocysteine levels measured at baseline. MTX: Methotrexate.

GGH promoter region may result in increased GGH expression/activity and decreased accumulation of MTXPGs eventually leading to reduced MTX sensitivity or drug resistance [24,32,33]. Supporting this observation studies in RA patients have found correlation between GGH 401T allele with reduced efficacy [34] and lower MTXPG accumulation [24]. We observed GGH 401TT was associated with hepatic and overall AE in our RA patients. One possible explanation is that lower levels of polyglutamation may cause cells to efflux excess MTX, thus leading to higher levels of MTX in the plasma and MTX-related AE. Alternatively, these patients might need a higher MTX dose to maintain the intracellular MTX polyglutamate pools in order to achieve therapeutic effect of MTX, and these high MTX doses might cause AEs. Our observation that the patients having GGH 401 TT genotype were receiving higher doses of MTX and showed higher AE could support the above hypothesis. However, PK analysis did not reveal association of this SNP with higher MTX plasma levels indicating MTX plasma measurements will be less informative in these patients and assessing MTX polyglutamate levels in these patients could be more helpful to explain the association of GGH 401TT with AE observed in our study.

It has been observed that carriers of a double 28-bp tandem repeat in the *TS* promoter exhibit lower *TS* gene expression and activity than those with triple 28-bp repeats [35]. Several studies exploring potential influence of *TS* 5'UTR repeats on MTX toxicity and efficacy in RA patients found patients homozygous for *TS* 5'UTR 3R allele required higher MTX dose and had poor response to MTX as compared with those with at least

one 2R [23,36,37]. When 2R/2R genotype was incorporated in toxicogenetic index together with several other folate cycle polymorphisms, significant correlation with toxicity to MTX was seen [25]. Similarly, we observed presence of *TS* 5'UTR*2/*2 genotype was associated with the MTX-related bone marrow AE in our RA cohort. Moreover, a 6 bp deletion in the 3'UTR of the *TS* has been shown to affect *TS* RNA expression, stability and has a significant association with poor outcome in 5 FU treated patients. Very few studies have reported effect of *TS* 3'UTR 6bp deletion on MTX response [11,23]. We find a significant association of *TS* 3UTR 6bp/6bp genotype with overall AE related to MTX.

SHMT1 encodes a vitamin B6 dependent enzyme that plays a vital role in delivering 1-carbon units for purine and thymidylate synthesis. A 1420C>T polymorphism in this gene influencing red blood cell folate levels has been described and carriers of the 1420CC genotype have reduced plasma and RBC folate levels compared with those with the 1204CT and TT genotypes [38]. We report presence of at least one SHMT1 1420C allele was associated with gastrointestinal (pain in abdomen) AE in this study. In agreement with our observations Weisman *et al.* demonstrated significant correlation of this SNP with MTX-related AE [25].

Folate is an essential cofactor for nucleotide and methionine synthesis that plays a vital role in normal cell growth, replication and has epigenetic influences [14]. It is possible that common polymorphisms producing subtle alterations in a key enzymatic stage are likely to present minimal effects in its homozygous variant form and still may get transmitted across generations [25]. With this background, a combined index was generated as the

Figure 5. Correlation of plasma levels of methotrexate measured at 2 h with adverse event (or adverse drug reaction) and toxicogenetic index. Scatter plot demonstrates correlation of plasma levels of MTX measured at 2 h with ADR and toxicogenetic index. As MTX dose increases, adverse events (squares) and toxicogenetic index (colored squares and upright triangles) increase.

ADR: Adverse drug reaction; MTX: Methotrexate; TI: Toxicogenetic index.

sum of homozygous risk genotypes (TS 5'UTR *2R/*2R + TS 6bp/6bp + GGH-401 TT + SHMT1 CC) carried by the patients. It was observed that each time addition of new risk genotype to the index was associated with an incremental increase in the risk of presenting with AE, and patients with an index of 4 (having all risk genotypes) were ~threefold more likely to experience a side-effect than those with an index of 0. We also evaluated synergistic effects of concurrent administration of corticosteroids, DMARDs or NSAIDs on the toxicity of MTX, and found no association of these concurrent drug administrations with AE. In addition when cumulative effect of risk genotypes in the form of TI was considered, we observed that the occurrence of MTX toxicity remained associated with the risk of genotypes in the subset of patients receiving MTX only and was not associated with concurrent administration of corticosteroids, DMARDs or NSAIDs.

MTHFR C677T (rs1801133) and A1298C (rs1801131) are the most extensively studied SNPs as far as toxicity or efficacy of MTX is concerned; though inconsistent associations have been reported. Similar to our result, Dutch and Japanese RA cohorts showed association of *MTHFR* 1298 A allele with greater clinical improvement with MTX [39,40]. However, comparable to other studies [41,42] we report negative associa-

tion of these SNPs with MTX-related toxicity in our RA population. The *RFC1* 80G/A nonsynonymous SNP rs1051266 results in substitution of arginine for histidine at codon 27 at the first transmembrane domain of the RFC1 protein [43] and may affect MTX transport into the cell and thus intracellular MTX-PG levels [24]. Meta-analysis of several independent observational studies reported the role of this SNP in terms of MTX-related efficacy [44]. Our results replicate the previous findings of association of this SNP with MTX-related efficacy in Indian RA patients. Thus these variants in *MTHFR* and *RFC1* hold promise as a potential predictor of MTX efficacy and should be assessed in prospective pharmacogenetic studies.

There are some limitations of this study. There was heterogeneity in the patient's cohort for this study in terms of different MTX doses and data regarding folic acid supplementation in these patients was not available. It is known that routine administration of folic acid in MTX taking patients can have effect on reduction of MTX-related AE. Second, we did not measure circulating intracellular levels of MTX polyglutamates in erythrocytes and polymorphonuclear cells in these patients which have been shown to correlate with clinical efficacy in patients with RA [45–47]. Studies have shown that the increased RBC long-chain MTXPG concentrations were

associated with a lower number of tender and swollen joints, a lower score for the physician's global assessment of disease activity and indicated that the patients with higher RBC MTXPG levels were more likely to have a good response to MTX and those with low MTXPG levels may need more aggressive MTX treatment to maximize polyglutamation and achieve efficacy [36,48]. It was further observed that patients with a lesser decrease in the DAS28 (fewer improvements) had lower RBC MTXPG levels ($p < 0.05$) despite the higher MTX dose administered [46]. Based on the above data, one can presume that the lower MTX efficacy in our cohort in part could be due to the presence of lower RBC MTXPG levels in these patients and further prospective studies are needed to postulate role of circulating intracellular levels of MTX polyglutamates in these patients. Third, the pharmacokinetic studies were done on relatively small sample size with limited time points. However, it is practically and economically challenging to obtain rigorously characterized PK end points in a larger cohort. To the best of our knowledge this is a first report on pharmacogenetics of MTX in Indians (Asian) RA patients covering 12 SNPs in nine candidate genes in folate-MTX pathway along with pharmacokinetic data. Results from our study replicate some previous findings from the literature and we also report some new associations between SNPs in the folate-MTX metabolic pathway with MTX response (toxicity and efficacy) in our RA cohort. In our

pilot study, we may have missed these associations due to very small sample size ($n = 34$) [13]. Our observations propose that a toxicogenetic index can provide a method of profiling patients who develop AE to MTX and may be useful in establishing the probability of occurrence of adverse effects to MTX. This could reduce the cost of drug monitoring and maximize the risk-benefit ratio of the drug.

Conclusion & future perspective

In summary, our study evaluated the influence of genetic variation in nine genes involved in the folate-MTX pathway genes with MTX response and its PK in Indian (Asian) patients with RA. Based on our results, we propose that the SNPs in the folate metabolic pathway can be used as predictive marker of MTX response; future studies in larger patient cohorts will be helpful to validate our findings.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/PGS.15.145

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Figure 6. Correlation of plasma levels of homocysteine measured at baseline with adverse event (or adverse drug reaction) and toxicogenetic index. Scatter plot demonstrates correlation of plasma levels of Hcy measured at baseline with ADR and toxicogenetic index. Dotted red line indicates range for normal Hcy levels (between 5 to 15 μ moles/l). Patients with high plasma Hcy levels (<15 μ moles/l) had higher adverse events (squares) and toxicogenetic index (red squares) than those with ≥ 15 μ moles/l plasma Hcy. ADR: Adverse drug reaction; Hcy: Homocysteine; TI: Toxicogenetic index.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Methotrexate (MTX) is a widely prescribed, cost-effective disease modifying antirheumatic drug for the treatment of rheumatoid arthritis (RA).
- Its use, however, is limited by wide interpatient variability in clinical response and unpredictable adverse events.
- Genetic variation in folate-MTX metabolic pathway genes likely contributes, in part, to interpatient differences in response.

Study objective

- The objective of this study was to use a pathway-driven approach for evaluation of association of genetic variants in folate-MTX pathway genes with MTX response (both toxicity and efficacy) and plasma MTX, 7OH-MTX and homocysteine (Hcy) measurements in Indian (Asian) patients with RA.

Results

- Out of 12 SNPs analyzed, SNPs in *GGH* promoter; *SHMT1*; *TS* 5'UTR variable tandem repeats and *TS* 3'UTR deletion was associated with MTX-related adverse event either organ specific or overall adverse event while SNPs in *MTHFR* and *RFC1/SLC19A1* were associated with MTX efficacy. *TS* 5'UTR and *SHMT1* polymorphisms were associated with higher plasma levels of MTX. A significant correlation was found between the SNP in *RFC1* and plasma Hcy levels in these RA patients.
- To the best of our knowledge, this is a first report on pharmacogenetics of MTX in Indians (Asian) RA patients covering 12 SNPs in nine candidate genes in folate-MTX pathway along with pharmacokinetic data.

Conclusion

- Our study evaluated the influence of genetic variation in nine genes involved in the folate-MTX metabolic pathway with MTX response and plasma levels in Indian RA patients. Based on our results, we propose that the SNPs in the folate metabolic pathway can be used as predictive marker of MTX response.

Future perspective

- Future studies in larger patient cohorts are required to validate our findings and better understand clinical implication of MTX pharmacogenomics.

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